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Estrogenic and anti-proliferative activity of water extract of *Glycyrhizzic uralensis* (licorice root)

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Abstract

Licorice is the root of *Glycyrrhiza uralensis*, which is a commonly used herb in traditional Chinese medicine. Licorice extract has been claimed to have anti-cancer, anti-viral, anti-inflammatory and anti-diabetic activities. This study evaluated the estrogenic effect of licorice root water extract on Ishikawa cells and related it to its effect on cell proliferation using various cell lines in comparison to glycyrrhizic acid (GA). This study showed that licorice root extract exhibited estrogenic property while decreasing cell proliferation thus suggesting possible use in estrogen replacement agent with anti-proliferative property. Even though GA is the most studied active component in licorice, these two properties were not seen in the cell lines tested; hence suggesting that GA is not responsible for these activities. These results showed that licorice could be further studied as an estrogen replacement agent as it does not cause an increase in cell proliferation in the breast and uterus.

Keywords: Glycyrrhizic acid, *Glycyrrhiza uralensis,* Health functional food, Licorice root, Phytoestrogen

Introduction

Licorice, which is the root of *Glycyrrhiza uralensis* (*G. uralensis*), is a commonly used herb in traditional Chinese medicine for the treatment of menopausal symptoms[1]. Licorice root has been shown to have anti-cancer [2], anti-viral [3] and anti-inflammatory [4]. Previous studies have shown that licorice root extracts have estrogenic activity towards estrogen receptors (ER)[5]. As licorice roots are a rich source of prenylated flavonoids [6], they might offer opportunities for its development into supplements aimed at women with menopausal symptoms. Despite the wide use of licorice root for the treatment of menopausal symptoms, little is known on its potential estrogenic properties and available information relative to its effects on cell proliferation is contradictory[7].

The principal bioactive components in licorice root are licochalone A, glycyrrhizin and glycyrrhizic acid (GA) [8], with GA being the most studied [9]. The plant contains phytoestrogens in the form of isoflavones. These phytoestrogens are a diverse group of non-steroidal plant-derived compounds that structurally or functionally mimic mammalian estrogens[10, 11]. They exhibit estrogenic activity in the body by binding weakly to ER to induce transcription of estrogen-responsive target genes in a dose-dependent manner [12, 13]. The relative affinities of phytoestrogens for ER are more than 10^2 to 10^5-fold lower than 17β-estradiol (17β-E_2) [14],which is an important factor when considering the dietary intake of phytoestrogens and their subsequent circulating concentrations [15]. Overall, data on the estrogenic actions of phytoestrogens is complexed by multiple factors such as the chemical structure of phytoestrogen, site of action, cell type, route of administration, metabolism, presence of endogenous estrogens and other treatments used in conjunction with phytoestrogens [13, 16].

At present no studied had compared the estrogenicity of licorice root water extract on various breast and endometrial cell lines in comparison to that of GA and 17β-E_2. Thus, in this study, the estrogenic effect of licorice root will be evaluated together with its effect on cell proliferation to determine if it could serve as a safe estrogen replacement agent. The licorice root extract will be tested alongside GA to determine if its main bioactive compound is responsible for these activities.

Materials and Methods

Chemicals

Minimum Essential Medium (MEM), dextran-coated charcoal (DCC) and 17β-Estradiol (17β-E_2) were purchased from Sigma, USA. RPMI-1640, fetal bovine serum (FBS), fetal calf serum (FCS), trypsin, antibiotic-antimycotic and L-glutamine were obtained from Gibco, USA. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) was sourced from Nacalai

Tesque, Japan. All other chemicals were purchased from Merck, Germany.

Cell culture and maintenance of Ishikawa cells

Human endometrial adenocarcinoma (Ishikawa) cells (99040201, Sigma) were maintained in MEM. Human breast adenocarcinoma (MCF-7) cells (ATCC HTB-22™) and human breast carcinoma (MDA-MD-231) cells (ATCC HTB-26™) were maintained in RPMI-1640. The media were supplemented with 10% FBS, 1% antibiotic-antimycotic and 1% L-glutamine. Cells were passaged twice weekly. Near-confluent cells were changed to an estrogen-free basal medium (EFBM). After 24 h, the cells were harvested with 0.25% EDTA-trypsin and seeded in 96-well flat-bottomed microtiter plates, in 100 µl of EFBM/ well. The EFBM consist of DMEM/ F-12 (Nacalai Tesque) media supplemented with 5% DCC-stripped FCS, 1% antibiotic-antimycotic and 1% L-glutamine.

Preparation of test compounds

The species of licorice root used was *G. uralensis*. Ten g of licorice root was boiled in 100ml of ultra pure water at 90 C for 10 mins. The decoction was filtered, freeze-dried and stored at -20 C until use. The licorice root extract was prepared as a 10mg/ml stock solution in water. The extract was tested against 10^{-8}M 17β-E_2, dissolved in ethanol (EtOH) (Merck) as the carrier. The stock solutions were then filtered with a 0.22µm filter to remove any precipitate and diluted to the appropriate concentrations in media for use. Concentration of EtOH for all samples used was maintained at 0.1%. All test compounds were stored at 20 C.

Alkaline phosphatase activity assay

Ishikawa cells were seeded in a 96-well flat bottomed plate at a density of 2.5x10^4cells/100 µL in EFBM/well. After 72h of incubation with test compounds, the wells were washed twice with ice-cold phosphate buffered saline (PBS). The plate was then frozen in -80 C for 20min before being thawed at 37^{0}C. After that, 50µL of ice-cold *p*-nitrophenyl phosphate (pNPP) (SIGMA) solution was added and the alkaline phosphatase (ALP) enzyme activity was monitored for 1.5 – 3h by reading the plate periodically at 405nm until maximally stimulated cells show an absorbance of about 1.2 [17]. All experimental conditions were assayed in triplicate.

MTT Cell proliferation assay

The effect of the extracts on cell proliferation were estimated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay, the cells were seeded at in a 96-well flat bottomed plate at a density of 1.5 10^4cells/100µL in EFBM/well 24h before addition of test compounds. After incubation with test compounds for 72 h, the 10µL of 5mg/ml MTT (Amresco, USA) was added to each well. Dimethyl sulfoxide (DMSO) was then added to dissolve the formazan and the plate read at 595nm [18]. The cell proliferation of control cells was 100.00% and any increase or decrease in cell proliferation of treated cells was compared to the control cells. Hence the untreated control was represented by a baseline of 0% cell proliferation above control [19]. All experimental conditions were assayed in triplicate.

Scratch wound assay

Ishikawa and MCF-7 cells were plated in 6-well plates. At 100% confluence, cells were scratched with a 100 µL pipette tip. The wounded cells were treated with EFBM consisting of the appropriate concentration of licorice root extract. The scratched region was photographed with a Nikon camera fitted to a microscope eyepiece at 24h, 48h and 72h after scratching. The gaps were then measured and calculated into % wound closure[20]. Results represent the average of three experiments.

Statistical analysis

All data were be expressed as mean ± SEM (Standard Error of Mean). Data collected were analyzed using a statistical software SPSS version 16.0 (SPSS Inc., USA).

Results and Discussions

Ishikawa cells responded to both estrogens and anti-estrogens at a concentration approximating physiological levels. They are very sensitive to estrogens and the effect is dose dependent [21]; detecting 17β-E_2 at concentration as low as 10^{-12} M [17]. Past studies had demonstrated that Ishikawa cells responded to estrogen administration with an increase in cell number and ALP activity, which can be inhibited by anti-estrogens [17, 21]. ALP expression is strictly under estrogenic control at the transcriptional level [22] and steroids such as androgens, glucocorticoids or progestins do not produce a similar effect [17].

ALP assay was used as the main assay for evaluating estrogenic activity of the compounds while MTT assay was performed to determine their effect on cell proliferation. The result showed that control cells had a low baseline level of ALP activity (0.25 ± 0.01A) and 17β-E_2 exhibited a dose-dependent stimulation of ALP activity in Ishikawa cells, with the maximum response at 10^{-8} M 17β-E_2 (1.22 ± 0.00A). Based on the results, stimulation of ALP activity by E_2 was similar to past studies [21, 23] where the effect is dose dependent and the maximum response was found at 10^{-8} M 17β-E_2 (1.22 ± 0.00A) which was 5-fold of that induced by the untreated control (0.25 ± 0.01A). While GA resulted in ALP activity similar to that of control cells, increasing concentrations of licorice root extract induced increasing estrogenic activity in Ishikawa cells (Figure 1). This showed that while GA is the most studied component of licorice, it did not display estrogenic activity.

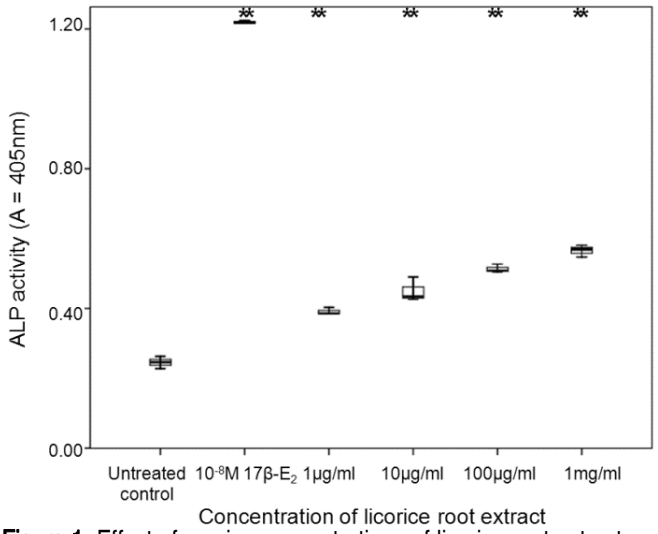


Figure 1: Effect of varying concentrations of licorice root extract on ALP activity in Ishikawa cells in comparison to the untreated control and 10^{-8}M 17β-E_2. ** P≤0.01.

Treatment of 10^{-8}M 17β-E_2 significantly increased cell proliferation in Ishikawa and MCF-7 cells from control cells (Figure 2). However as with Berthois *et al.*, treatment of 10^{-8}M 17β-E_2 did not have any effect on the cell proliferation of MDA-MB-231[24]. Cells grew at almost the same rate as control cells. This is because MDA-MB-231 is an ER negative cell line and is therefore estrogen and anti-estrogen unresponsive[24], as compared to Ishikawa and MCF-7 cells who are both ER positive [25, 26].

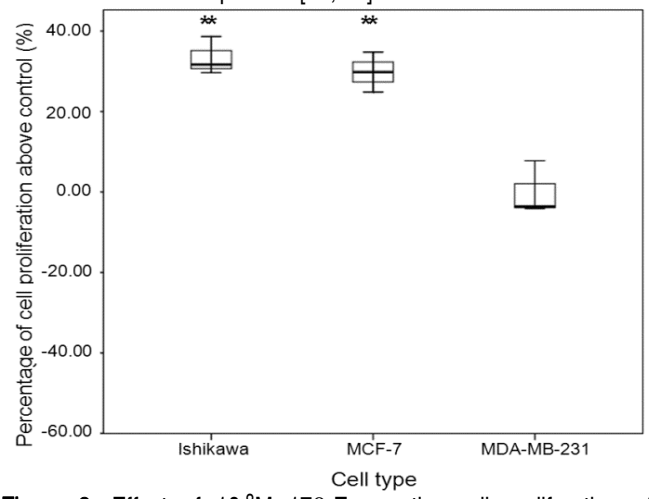
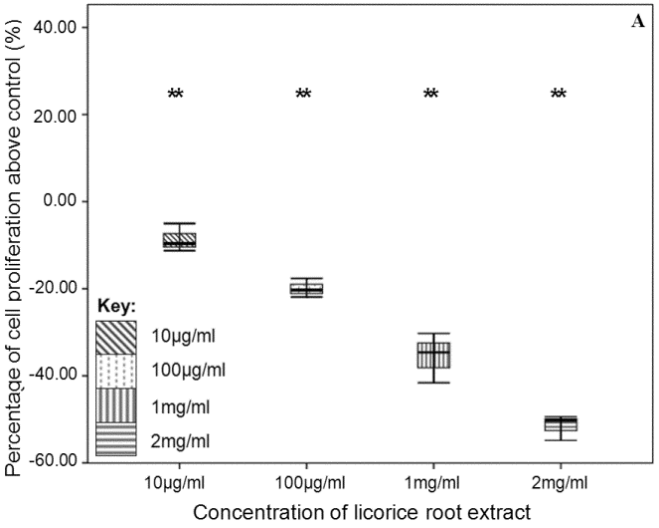
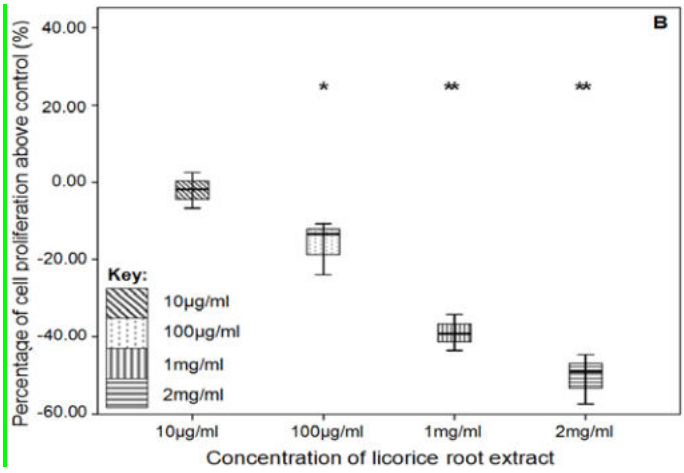


Figure 2: Effect of 10^{-8}M 17β-E_2 on the cell proliferation of Ishikawa, MCF-7 and MDA-MB-231. ** P≤0.01.

In Ishikawa (maximal inhibitory concentration (IC_{50})= 1.94mg/ml) and MCF-7 cells (IC_{50}= 1.89mg/ml), increasing concentrations of licorice root resulted in significant decrease in cell proliferation (Figure 3A and 3B, respectively) as compared to GA which did not significantly decrease cell proliferation (results not shown). However, licorice root extract did not exhibit any effect on MDA-MB-231 whereby, the cell proliferation rate was similar to that of control cells (Figure 3C). The decrease in cell proliferation observed in Ishikawa and MCF-7 could be due to phytoestrogens present in the licorice root extract, as shown by the ALP assay, which act through ER to decrease cell proliferation.





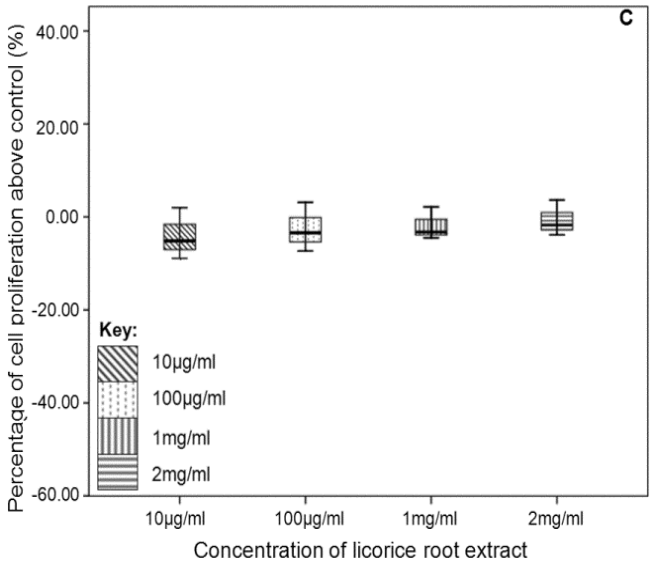


Figure 3: Effect of varying concentrations of licorice root extract on the cell proliferation of Ishikawa (A), MCF-7(B) and MDA-MB-231(C) respectively. *P≤0.05 and ** P≤0.01.

Phytoestrogens include phenolic compounds which exhibits high estrogenic activity while preventing increase in cell proliferation[27]. These are positive effects in comparison to estrogen replacement therapy (ERT) drugs which increases risk of cancer. This study showed that licorice extract could be further studied to identify active phytoestrogenic compounds which could be used as an ERT without the side effects of ERT.

Past studies have also shown the estrogenic effects of licorice root in Ishikawa cells and MCF-7cells, however those studies were done with organic solvent extracts such as methanol, DMSO or hexane[7, 28]. The results obtained from this experiment was different from a past study by Hu *et al.*, which showed that water extracts of licorice root stimulated a dose-dependent increase in cell proliferation of MCF-7 cells from 1-500ug/ml. The study also showed that the increase in cell proliferation was not inhibited by anti-estrogens concluding that the increase was ER-independent [7].

For the scratch assay, Ishikawa and MCF-7 tested responded similarly with a decrease in cell migration, compared to control cells. The decrease in cell migration in licorice root treated cells again supports its use as a safe agent that does not induce cell proliferation or cancer risk. After 24h, Ishikawa control cells closed the wound 72.73±10.5% while licorice root extract treated cells closed only 59.26±9.8% of the wound. As for MCF-7 cells, control cells closed 45.00±2.89% and licorice root extract treated cells closed only 35.90±6.78% of the wound (Figure 4).

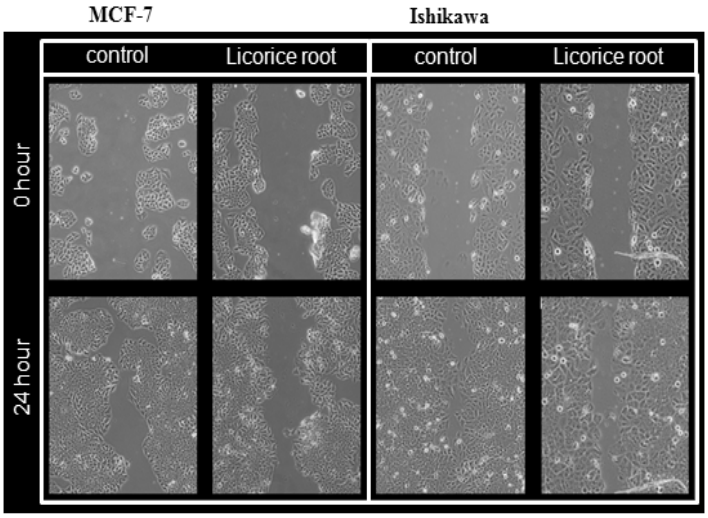


Figure 4: Effect of 1mg/ml of GA and licorice root water extract on the cell migration of Ishikawa cells and MCF-7 in comparison to the untreated control.

ER-dependent effects of licorice root had been attributed to presence of glabrene and glabridin which could be isolated from organic solvent extracts of licorice root such as acetone [29, 30]. However, based on the ethno botanical use of licorice root, it is usually boiled in water and then consumed. Therefore this study used a water extract of licorice root to determine if it provides phytoestrogenic benefits through its traditional use. Further studies could be done to identify the bioactive components presents and to determine the amount of glabrene or glabridin present in the water extract.

Conclusion

Based on the results, licorice extract demonstrated dose-dependent estrogenic effect on Ishikawa cells. This showed that it could be further studied to identify other active compounds which could be used as a possible estrogen replacement agent without the side effects of conventional ERT. This is further supported by the decrease in cell proliferation induced by licorice root in Ishikawa and MCF-7 cells. Further studies include the identification of phytoestrogenic bioactive compounds in licorice root and the exploration of cell proliferation inhibition mechanism of licorice root extract through ER.

Author's Contributions

MPSW carried out all the assays, statistical analysis described in this paper and drafted the manuscript. CYY helped concepted the study, revised and approved the final manuscript for publication.

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